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CELL AND ORGANOID MODELS TO DEVELOP NEW ANTIVIRALS AND DIAGNOSTIC METHODS FOR EMERGING VIRUSES

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Cell and organoid models to develop new antivirals and diagnostic methods for emerging viruses

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Instead of thinking what you want to do, think about who you want to be.

- Jayson Demers

POPULAR SCIENCE SUMMARY OF THE THESIS

Virus outbreaks have always been a threat to mankind. The Spanish flu at the beginning of the 20th century, the Zika virus outbreak in the Americas in 2015, or the Ebola virus outbreak in 2016 in West Africa are only a few examples. Likewise, the ongoing COVID-19 pandemic has brought unprecedented social and economic disruptions to countries all over the globe and has brought to public attention that we are not well prepared for new virus outbreaks. In particular medicines are missing to treat infected people, as well as easily adaptable diagnostic tests, to enable fast set up of large-scale testing. Fast diagnosis is not only important to diagnose a virus disease in a patient, but also to assess the spread in the population and steer the response to a pandemic.

A big obstacle to the development of medication and diagnostic tests for viruses is the lack of good models to study viruses. In biology models are substitute organisms that are studied extensively to understand biological phenomena, human disease or in the case of pathogens, understand the behavior and effects of pathogens in humans. Models are widely used in research, because it can be unethical or unfeasible to do certain experiments in humans. The underlying assumption is that the findings from models are transferable to human disease. However, what complicates matters for virus research is that viruses can be very selective about the species they infect, and the disease viruses cause is not the same in all species. The work from this thesis highlights how different models can be used to speed up the development of new medication and diagnostic tools for viruses.

When viruses infect cells, they create certain observable traits, also called phenotypes. For example, the virus can be detected inside cells using fluorescent antibodies, and thus infected and uninfected cells can be distinguished and counted when images of the cells are taken. This principle was used in the first paper included in the thesis. A total of 425 compounds were tested for their antiviral properties by checking if they reduce signal from fluorescent antibodies against virus inside infected cells. This way, we found two substances with an antiviral effect. Subsequently, using the same method but different viruses, we confirmed that the two substances identified do not only work against a single virus. We discovered that the substances have activity against several viruses including Ebola and SARS-CoV-2 virus, the virus which is causing the COVID-19 pandemic.

The second paper focused on the action of the newly discovered antivirals against Zika virus. First, we confirmed that these substances were active against Zika virus. Zika virus can cause a small brain in children by infecting their brain cells, also called neurons, if their mother is infected with Zika virus during pregnancy. To mimic this, we used cells derived from human brain tumors to study the antiviral substances and confirmed that they have an antiviral effect against Zika virus.

However, the brain is the body's most complex organ. This complexity makes it very difficult to find a suitable model for the human brain. The brain tumor cells we used previously are very homogenous and one-dimensional, while the brain is a three-dimensional organ with many

different types of cells which communicate with each other. While this is difficult to model in traditional cell culture, in the past decade three-dimensional organoids have become available. It has been shown previously that brain organoids are a great model for Zika virus: just as in the fetal brain, Zika virus is able to infect neurons in the organoids and reduce their viability. We were able to show that our compounds not only reduce the amount of Zika virus in the organoids but also restore organoid viability.

The third study from this doctoral thesis focused on the development of a new diagnostic tool to detect Zika virus by multiplying parts of its genome. In the development of the diagnostic tool, both cell lines and blood cells from healthy donors were infected artificially in the lab to generate samples to test the new approach for sensitivity. Infecting blood cells from healthy donors with virus mimics the real infection in the patients, and the Zika virus levels found in the artificially infected samples resemble the ones found in patients. This approach helps research groups to develop diagnostic tools even without access to patient samples, which are not easy to acquire, especially for infections from remote regions or low-income countries.

Altogether, the first two studies highlight the use of different cell and organoid models to advance antiviral drug development. The third study focuses on developing new diagnostics for Zika virus using cells and human blood as a model. These approaches contribute to advancing the drug and diagnostics development and can help to be better prepared for future pandemics.

ABSTRACT

The ongoing global pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is only the latest reminder of the urgent need for new antiviral strategies and diagnostic tools to improve preparedness for emergence of new pathogenic RNA-viruses. A key obstacle for the development of antivirals and diagnostic tools has been the lack of good models to study RNA viruses. This thesis focused on cellular and organoid models for virus infection to facilitate the development of host-targeting antivirals and new diagnostic tools against emerging viruses.

In **Paper I**, two close analogues from an in-house library of 425 host-targeting compounds were found to have antiviral activity against apathogenic RNA virus Hazara virus (HAZV). These two hit compounds were discovered using a cell- and image-based phenotypic antiviral screening assay. Subsequently, the two compounds were confirmed to have a broad-spectrum antiviral activity against several pathogenic RNA viruses including SARS-CoV-2, Ebola and Crimean-Congo hemorrhagic fever virus. After showing the independence of the compound's antiviral effect of their originally designed target, human 8-oxoguanine glycosylase 1, thermal protein profiling was used to study the compound target. Applying this technique, the compound was revealed to disturb proteostasis pathways and interactions between cellular heat shock protein 70 complex and viral proteins.

In **Paper II**, a second antiviral screening of in-house compounds was performed using the previously established image-based phenotypic antiviral screening assay with Zika virus (ZIKV) infected cells. The broad-spectrum activity of the compounds was confirmed by revealing the same compounds to be active against both HAZV and ZIKV. Next, the therapeutic window and antiviral activity of the top-hit compounds was demonstrated in several cellular models of ZIKV infection. Additionally, the novel antiviral compounds showed an antiviral effect and reversed ZIKV induced neurotoxicity in iPS cell derived human brain organoids. Furthermore, mechanism-of-action studies revealed the compound to impair the formation of new virus particles in the late lifecycle steps.

In **Paper III,** the development of a new diagnostic tool for ZIKV is described using *in vitro* infected U87 cells and PBMCs. In the method presented, ZIKV cDNA was hybridized using padlock probes and amplified by two rounds of Circle-to-Circle Amplification. Detection was performed using a microfluidic affinity chromatography enrichment platform. Benchmarking of the newly developed method against RT-qPCR, the gold standard diagnostic method for ZIKV detection, confirmed a good correlation between both methods.

Altogether, this thesis demonstrates how cell culture tools with varying complexity, unique advantages and challenges can be used to augment the development of novel antiviral drugs and diagnostic methods.

LIST OF SCIENTIFIC PAPERS

Scientific Papers included in the thesis

I. **Novel Broad-Spectrum Antiviral Inhibitors Targeting Host Factors Essential for Replication of Pathogenic RNA Viruses.**
Tampere, M.; **Pettke, A.**; Salata, C.; Wallner, O.; Koolmeister, T.; Cazares-Körner, A.; Visnes, T.; Hesselman, M.C.; Kunold, E.; Wiita, E.; Kalderén, C.; Lightowler, M.; Jemth, A.-S.; Lehtiö, J.; Rosenquist, Å.; Warpman-Berglund, U.; Helleday, T.; Mirazimi, A.; Jafari, R.; Puumalainen, M.-R.
Viruses 2020, 12, 1423.

II. **Broadly Active Antiviral Compounds Disturb Zika Virus Progeny Release Rescuing Virus-Induced Toxicity in Brain Organoids.**
Pettke, A.*; Tampere, M.*; Pronk, R.; Wallner, O.; Falk, A.; Warpman Berglund, U.; Helleday, T.; Mirazimi, A.; Puumalainen, M.-R.
Viruses 2021, 13, 37.

III. **Circle-to-circle amplification coupled with microfluidic affinity chromatography enrichment for in vitro molecular diagnostics of Zika fever and analysis of anti-flaviviral drug efficacy.**
Soares, R.; **Pettke, A.,** Robles-Remacho, A.; Zeebaree, S.; Ciftci, S.; Tampere, M.; Russom, A.; Puumalainen, M.-R.; Nilsson, M.; Madaboosi, N.
Sensors and Actuators B: Chemical, Volume 336, 1 June 2021, 129723

Scientific Papers not included in the thesis

IV. **Managing COVID-19 in the oncology clinic and avoiding the distraction effect**
Cortiula, F.; **Pettke, A.**; Bartoletti, M.; Puglisi, F.; Helleday, T.
Annals of Oncology, 2020 May;31(5):553-555. (Editorial)

V. **Targeting OGG1 arrests cancer cell proliferation by inducing replication stress**
Visnes, T.*; Benítez-Buelga, C.*; Cázares-Körner, A.; Sanjiv, K.; Hanna, B.M.F.; Mortusewicz, O.; Rajagopal, V.; Albers, J.J.; Hagey, D.W.; Bekkhus, T.; Eshtad, S.; Baquero, J.M.; Masuyer, G.; Wallner, O.; Müller, S.; Pham, T.; Göktürk, C.; Rasti, A.; Suman, S.; Torres-Ruiz, R.; Sarno, A.; Wiita, E.; Homan, E.J.; Karsten, S.; Marimuthu, K.; Michel, M.; Koolmeister, T.; Scobie, M.; Loseva , O.; Almlöf, I.; Unterlass, J.E.; **Pettke, A.**; Boström, J.; Pandey, M.; Gad, H.; Herr, P.; Jemth, A.-S.; El Andaloussi, S.; Kalderén, C.; Rodriguez-Perales, S.; Benítez, J.; Krokan, H.E.; Altun, M.; Stenmark, P.; Warpman Berglund, U.; Helleday, T.
Nucleic Acids Research 2020 Dec 2;48(21):12234-12251.

VI. **A rapid phenomics workflow for the in vitro identification of antiviral drugs**
Rietdijk, J.; Tampere, M.; **Pettke, A.**; Georgiev, P.; Lapins, M.; Warpman Berglund, U.; Spjuth, O.; Puumalainen, M.-R.; Carreras-Puigvert, J.
Preprint on bioRxiv; https://doi.org/10.1101/2021.01.13.423947

VII. **Long-term SARS-CoV-2-specific and cross-reactive cellular immune responses and their correlates with humoral responses, disease severity and symptomatology**
Laurén, I.*; Havervall, S.*; Ng, H.; Lord, M.; **Pettke, A.**; Greilert-Norin, N.; Gabrielsson, L.; Chourlia, A.; Amoêdo-Leite, C.; Josyula, V.S.; Eltahir, M.; Kerzeli, I.; Jernbom Falk, A.; Hober, J.; Christ, W.; Wiberg, A.; Hedhammar, M.; Tegel, H.; Burman, J.; Xu, F.; Pin, E.; Månberg, A.; Klingström, J.; Christoffersson, G.; Hober, S.; Nilsson, P.; Philipson, M.; Dönnes, P.; Lindsay, R.*; Mangsbo S.*; Thålin, C.*
Manuscript under revision

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CONTENTS

LIST OF ABBREVIATIONS

AI	Artificial intelligence
CZD	Congenital Zika Disease
COVID-19	Coronavirus diseases 2019
CoV 229E	Coronavirus 229E
C2CA	Circle-to-Circle Amplification
C2CA-μACE	Microfluidic affinity chromatography enrichment
EBOV	Ebola virus
HAZV	Hazara virus
iPS cells	Induced pluripotent stem cells
LAMP	Loop-mediated isothermal amplification
LoD	Limit of detection
NHPs	Nonhuman Primates
OGG1	8-oxoguanine glycosylase 1
PBMCs	Peripheral blood mononuclear cells
RCA	Rolling circle amplification
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
3D	Three-dimensional
2D	Two-dimensional
ZIKV	Zika virus

1 INTRODUCTION

1.1 GENERAL INTRODUCTION TO EMERGING VIRUSES

The currently ongoing global pandemic of Coronavirus diseases 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is only the latest example of the significant public health threat posed by emerging pathogens. In the last decade, we have seen outbreaks of several pathogenic RNA viruses like SARS causing pneumonia, Ebola virus (EBOV) causing lethal hemorrhagic fever and Zika virus (ZIKV) causing congenital Zika syndrome (CZS), to name just a few prominent examples. Viral emergence is driven by increasing urbanization, rapid population growth, increased travelling activities, as well as climate change and global warming. All of these man-made problems entail a spread of vectors to previously unaffected areas.

Common denominators of emerging RNA viruses are the lack of vaccines, available therapies and in-depth knowledge about the pathogenesis, host reactions and underlying molecular events. New insights and approaches are needed to develop therapeutic strategies towards these inevitable global threats.

1.2 ZIKA VIRUS

1.2.1 Epidemiology & outbreaks

ZIKV was first isolated in 1947 in Uganda (1). It is an arbovirus and belongs to Flaviviridae family (2). Mosquito species *Aedes aegypti* and *Aedes albopictus* are natural vectors of ZIKV infection, therefore ZIKV spread is closely linked to the spread of these vectors in the tropical regions of the world, between 23.5°S and 23.5°N latitude (3). However, several other transmission modes have also been reported for ZIKV, including blood transfusions (4), sexual intercourse (5) and maternal-fetal transmission (6).

For decades after it was first isolated, ZIKV was associated with few cases of very mild human disease, until it caused outbreaks in Yap state, Micronesia in 2007 (7) and subsequently a much bigger outbreak in French Polynesia in 2013, with more than 30,000 people infected (8,9). During this outbreak ZIKV was linked to serious neurological complications for the first time (10,11). From French Polynesia, the virus was brought to Brazil by travel, most likely in the end of 2014. The combination of a previously unexposed and thus unprotected population and other factors like the low activity in vector control measures led to an exploding epidemic with ca. 1.5 million people infected (12,13).

Given its described evolution, ZIKV is a particularly interesting and serious example of viral emergence, and the transition of a “harmless” and presumably unimportant pathogen to a public health threat on a global scale.

1.2.2 Clinical implications of ZIKV infection

For the majority of patients, ZIKV infection remains harmless: most cases are believed to be asymptomatic or cause a self-limiting, very mild disease (14). In these patients the most common symptoms include fever, conjunctivitis, headache, pruriginous maculo-papular rash, arthralgia and myalgia (2,15,16).

Nevertheless, even asymptomatic and self-limiting diseases can be detrimental for women who are pregnant at the time of ZIKV infection, because of the dramatic effect for the fetus (17). ZIKV has been shown to be teratogenic, lead to miscarriages (18) and cause a disease complex named Congenital Zika Syndrome (CZS) (15). CZS collectively describes various neurological complications in newborns following ZIKV infection of the mother during pregnancy (19). These pathologies are the result of neuronal cell death in fetuses following ZIKV infection. The hallmark of CZS is microcephaly (20,21), a reduction in brain volume and subsequently also the cranial structures (22). CZS also features structural eye anomalies and posterior ocular lesions like chorioretinal and optic nerve atrophy (23,24), as well as congenital contractures (25).

However, studies have demonstrated that only 2.5-5% of babies born to mothers with ZIKV infection during their pregnancy develop birth defects (26,27). In the rest of the cases, the babies do not suffer from CZS, despite a maternal ZIKV infection during pregnancy. It is unclear if the maternal immune system is able to clear the infection prior to a transplacental infection of the fetus, or if despite the infection the fetuses do not develop disease. One contributing factor seems to be the stage of pregnancy at the time of infection. While up to 15% of babies of mothers infected during the first trimester develop neurological defects, the percentage declines in later pregnancy stages. Moreover, not all neuronal damage presents as CZS. Large long-term studies will be needed to assess if children with no clear CZS symptoms at birth might still suffer from more subtle signs of fetal neuronal damage like learning difficulties, mental health problems or social problems.

In adults, ZIKV infection has been linked to a serious neurological complication, named Guillain-Barré Syndrome (GBS). GBS is a polyneuropathy of autoimmune origin, and its onset is closely linked to previous infection by various pathogens (28) including ZIKV. The destruction of peripheral nerves causes facial palsy, distal limb paresthesia and muscle weakness, rapidly progressing to paralysis (29). In severe cases, paralysis of the respiratory muscles can result in breathing difficulties and the need for intensive care (30).

As of today, there is no cure or approved therapy for neither CZS nor GBS and patients are treated with supportive care.

1.2.3 ZIKV life cycle

To be able to develop new treatments, a deep knowledge of ZIKV life cycle steps and the molecular mechanisms underlying infection is needed. However, as of today, not all steps of ZIKV replication have been investigated in detail. Generally, ZIKV replication is thought to

be similar to other members of the Flaviviridae family and can be divided in the following steps: attachment and entry, translation of the viral RNA into proteins, replication of the virus genome, assembly and egress from the cell (31). For most of these steps viruses use and abuse various host cell factors and machineries, which determines their ability to infect and propagate in different tissues and cells, also called viral tropism.

ZIKV has been found to have a broad tissue tropism including skin, blood, brain, retina, placenta and testis (32–36). Notably, the susceptibility of these cells and tissues to ZIKV infections explains the pathology caused by ZIKV infection and its modes of transmission.

To enter any of these target cells, viruses need receptors which can directly or indirectly facilitate its incorporation to the cell. While the exact entry receptor for ZIKV is still unknown, several candidates are under discussion. Due to the previously mentioned similarities between Flaviviruses, research has focused on two distinct families of transmembrane phosphatidylserine receptors: TIM (TIM1, TIM3 and TIM4) and TAM (TYRO3, AXL and MER). Both of these receptor families are known to be important for DENV entry (37), and they are also known to regulate apoptosis and innate immune functions of host cells (38,39). However, contrary to initial reports of AXL being very important for ZIKV entry to the cells (40,41), several studies suggest that none of the receptors mentioned above is indispensable for the ZIKV entry process (42,43).

Following attachment to its yet-to-be-determined host cell receptor, ZIKV particles are internalized by endocytosis and trafficked to endosomes (44). The acidic environment in the endosome facilitates a conformational change of the flavivirus envelope protein prompting fusion of the viral and endosome membranes and release of the genome (45).

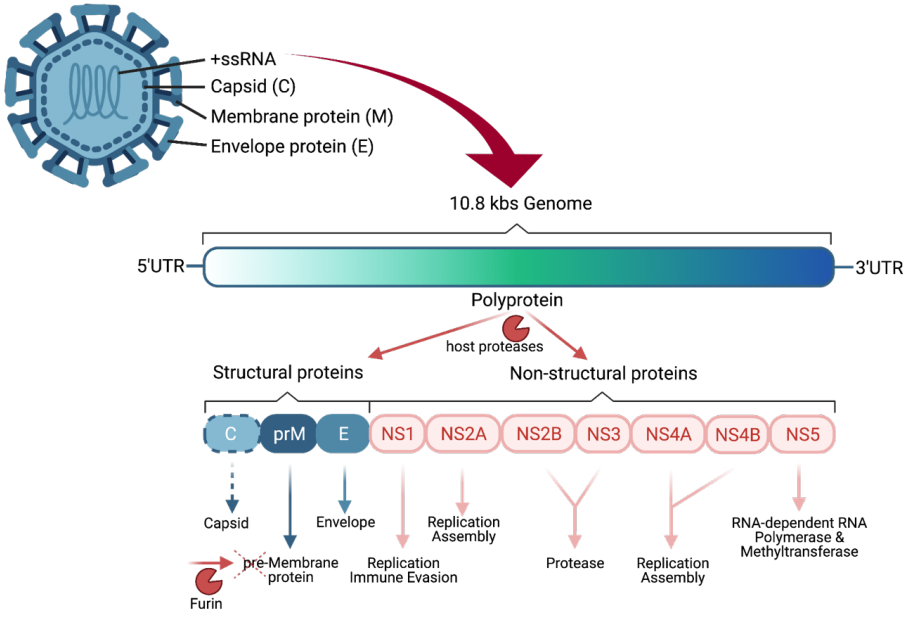

Figure 1: The structure of the ZIKV genome and its encoded proteins and their function in the viral life cycle

The ZIKV genome is a single stranded (ss) positive sense (+) RNA with a length of 10,794 kb, and it is organized in a single open reading frame. It codes for a polyprotein which is later cleaved proteolytically into three structural and seven non-structural proteins, which are important for viral replication (46) (Figure 1). The release of the genome is followed by a first translation step, which is crucial to produce all viral proteins needed for viral replication including the viral RNA-dependent RNA polymerase (RdRP) (NS5).

The RdRP initiates virus replication by synthesizing a (-)RNA intermediate from the complimentary (+)ssRNA. This (-)RNA is subsequently used as a template to generate many (+)ssRNA copies (47). Similar to other Flaviviruses, ZIKV replication takes place in so called replication factories (RFs) in the endoplasmic reticulum (48). The spatial segregation of viral and cellular compartments probably provides protection for the viral RNA from host nucleases and from detection by the innate immunity sensors. Through pores in the RFs the resulting (+)ssRNA is released into the cytosol (48), where it serves multiple purposes: production of new virus proteins, formation of new RFs and packaging into new virus particles.

The still immature and non-infectious virions are assembled in the ER, consisting of the E and prM proteins on a lipid membrane bilayer and the nucleocapsid in the core formed by C protein and RNA (49). To be able to undergo membrane fusion in the next cell, the virus particles need to mature (50,51). This happens during the transit through the trans-Golgi-network towards the cell surface, when the prM protein is cleaved to M by a Furin-like protease. In the final step, virus particles are released to the extracellular environment by exocytosis (Figure 2).

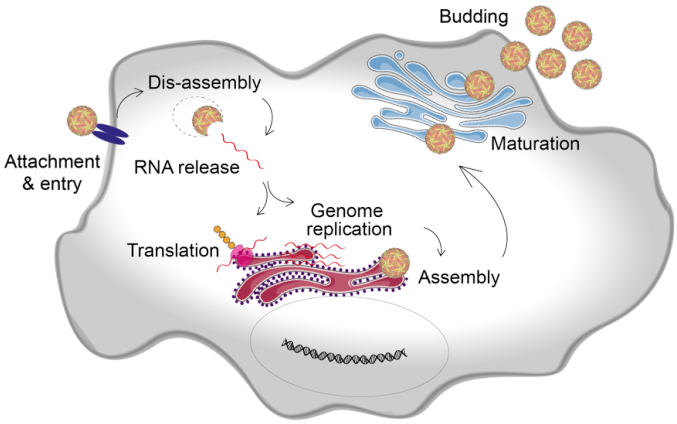

Figure 2: Schematic drawing of the ZIKV life cycle in a cell

1.3 ANTIVIRALS

The mortality and morbidity caused by emerging diseases is increasingly high (52–55), and new and more frequent outbreaks demonstrate how vulnerable the lack of vaccines and therapeutics leaves us in the face of viral emergence. While the development of highly efficacious and safe vaccines against SARS-CoV-2 within just one year, gives hope for future pandemics and redefines the limits of vaccine development (56), it does not make antivirals obsolete. New preferably broadly active antivirals are needed to bridge the time until vaccine development and deployment, and to provide treatment opportunities for individuals who cannot be vaccinated. This is why extensive drug discovery efforts are still needed to ameliorate the medical need and economic burden of emerging viruses.

1.3.1 Host targeting and direct antivirals

When fighting virus infections, two distinct therapeutic strategies can be implemented: crucial parts of the virus can be targeted directly (directly acting antivirals) or cellular proteins and machineries indispensable for virus replication can be targeted (host-directed antivirals). Both approaches come with advantages and drawbacks (57).

Directly acting antivirals are very specific. They target one particular viral protein, which comes with the disadvantage of needing to find and confirm a good, druggable viral target. In addition, it can also limit the utility of the newly identified antivirals to just one virus. Furthermore, a hallmark of RNA viruses like ZIKV is their high mutation rate leading to production of many genetic variants (quasi species) in every replication round (58). Directly targeting an important virus protein constitutes a high selection pressure, and results in the rapid selection of resistant virus variants. Combining several directly acting antivirals with distinct targets and increasing effectiveness of antivirals can help to reduce development of resistance, as has been shown very impressively by treatment regimens for human immunodeficiency virus type 1 (59). Also, by targeting the virus itself, less host impact and thus less side-effects can be expected.

Host targeting antivirals, on the other hand, can slow down the development of resistance, since it will require more time for the virus to adjust its entire replication machinery. Another advantage is the potentially broad antiviral activity. This is especially true for Flaviviruses, in which the replication process and use of host machinery is quite conserved. However, this comes at the cost of potential side effects, as a result of blocking pathways important for the host cell themselves (60,61). Furthermore, identifying the host target can be a long and challenging process.

An antiviral which has been used experimentally against various emerging viruses, including Lassa virus (62) and Crimean-Congo hemorrhagic fever virus (63) is ribavirin. Ribavirin is a guanosine analogue, with demonstrated broad spectrum activity against DNA and RNA viruses. Ribavirin's mode of action is not completely clear, but most likely it is a combination of direct effects from RdRP inhibition, interference with RNA capping, and from insertion of lethal mutations as well as indirect antiviral effects through immunomodulatory effects (64).

Ribavirin has also been reported to inhibit ZIKV infection *in vitro* in various cell models and in vivo (65,66).

1.3.2 Screening for new antivirals

In the past decade completely new approaches to drug screening have been developed and are becoming widely accessible. The increasing convenience and affordability of genome wide screening methods like RNA-interference, haploid cells, and the CRISPR technology (67,68) combined with the accelerated development in the field of computational biology and chemistry provides new ways and opportunities for drug screening (69).

Apart from the very new ways of screening, other approaches can be applied to identify new antiviral substances. In line with the two main antiviral strategies - virus- vs host-directed - a target-based approach and a phenotypic screening approach can be used, both of which provide unique opportunities and challenges.

Target-based screening approaches usually use a biochemical assay to test drug-candidates specifically for their activity against a predefined target. The use of a target-based approach provides the opportunity of rapid lead compound optimization, once a promising candidate is identified. However, there is no guarantee that an inhibitor identified in a biochemical assay will also work in cells.

Phenotypic screening assays are typically cell- or organism-based, which mean they primarily identify compounds which work in a physiological cellular context. This type of screening is largely unbiased, allows identification of both cellular and direct inhibitors and can be used to uncover new host-pathogen interactions as well as new drug targets with potentially broad-spectrum activity. However, for phenotypic screening approaches it is crucial which cell or organism model is chosen for the screening, because the performance of a compound might differ across cell types and species.

1.4 MODELS IN ZIKV RESEARCH

Independently of the screening approach, cellular and organism-based models are needed to confirm and optimize potential antiviral treatments, as well as study host and virus biology. However, model development is challenging. Viral tropism and species-specific interactions of viruses with the host lead to poor replication of many viruses in cellular models and differences in host reactions between humans and animals used in studies. The following chapter summarizes various systems developed to model ZIKV infection, to facilitate research on ZIKV and promote the development of antiviral therapies and vaccines.

1.4.1 Cell culture & organoids

Two-dimensional (2D) cell culture models are a cheap, established and highly controlled way to study many aspects of the virus life cycle as well as the host reaction, and they have been used intensively to study ZIKV (70). In a 2D monolayer, infections can spread very efficiently, since all cells are accessible to the virus at the same time. These models are very useful to study

viral tropism and show if the virus in question can infect the cells used as a model. Since many events in the virus life cycle are very similar in cells and organisms, results can often be extrapolated, and cell lines are a good starting point for antiviral drug development.

Vero cells, green monkey kidney cells, are a cellular model commonly used in the virology field, because they support the growth of many different viruses, they are used to isolate viruses and measure viral titers (71). Nevertheless, while they support the growth of ZIKV (72), they are not the most physiologically representative model. Keeping in mind that ZIKV infection primarily causes microcephaly in the developing brain, brain derived cell cultures represent a more relevant model. U87 cells are a glioblastoma cell line (73) which has been shown to sustain ZIKV infection, and also been used for some mechanistic studies, like studying inflammasome activation upon ZIKV infection (74).

Even if they are of a more relevant origin, U87 is a cancer cell line and two dimensional. In contrast to this, the human brain is an extraordinarily complex organ, and the interaction of various different cell types is crucial for its function. The complexity and cellular diversity of the human brain is one reason why there is a lack of accurate models to study the human brain. To further complicate matters, the remarkable qualitative and quantitative differences between animal and human brains e.g. between human and rodent brains (75), make the development of suitable animal models challenging.

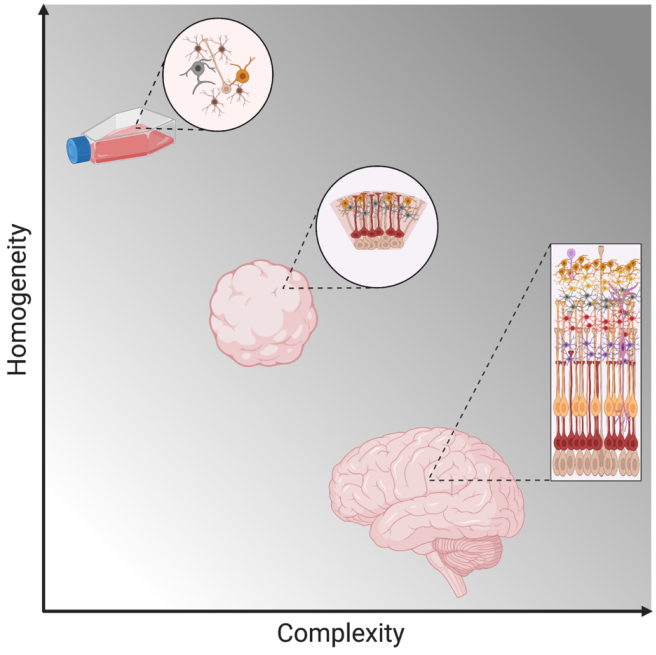

Figure 3: Schematic visualization of the complexity and homogeneity of cerebral organoids in relation to 2D cell cultures and the human brain.

To tackle this problem, three-dimensional (3D) cell cultures have recently been developed from skin-derived induced pluripotent stem (iPS) cells, to mimic more closely the natural physiological conditions (Figure 3), and especially the complex cytoarchitecture. These models have been used successfully to study microcephaly caused by ZIKV infection (76,77).

1.4.2 Blood

While conventional 2D and modern 3D cell cultures are good models to study virus pathogenesis and host interactions, a crucial drawback of cell lines is their genetic and phenotypic homogeneity. Moreover, many cell lines are derived from tumors and thus have intrinsically altered host pathways (78) and immune responses (79). Primary cells on the other hand resemble the original tissue quite well, however, they are not easily accessible and are difficult to work with.

Blood remains the most convenient, cost-effective and easy to work with primary tissue from either patients or healthy donors. Since ZIKV has been shown to infect peripheral blood mononuclear cells (PBMCs) (80), PBMCs have been used in research to model ZIKV infection and study host immune responses.

1.4.3 Animal models of ZIKV infection

While many features of virus infection are similar between cell culture, especially primary cells, and the organism as a whole, certain complex host defense strategies, such as the adaptive immune response, are not found in cell culture. Also, the main pathologies caused by ZIKV infection are serious neurological disorders in the fetus infected during pregnancy, another complex system which cannot be studied in cell culture. This highlights the need for a more sophisticated model to study ZIKV comprehensively.

Reflecting this necessity, several mammalian models of ZIKV infection have been developed since 2016. The most studied model organisms are immunodeficient mice (81), which can provide valuable insights into pathogenesis of ZIKV infection. However, the relevance and transferability of these data for the human disease are questionable, since their immune response upon infection is altered and it has been shown that susceptibility to infection largely differs with the age of these mice (82). Nonhuman Primates (NHPs), on the other hand, provide a much more relevant model, especially for ZIKV infection during pregnancy (83). Among the common features of ZIKV infection in humans and NHPs is vertical transmission from mother to fetus, as well as fetal abnormalities similar to CZD regardless of the severity of the maternal infection (84,85). However, drawbacks of using NHPs as disease models include ethical considerations, high costs and extended experimental time due to the relatively long duration of pregnancy.

A more cost- and time-efficient model to study ZIKV is the chicken embryo. Historically, chicken embryos have been a powerful tool to study developmental biology, embryology, as well as teratogenicity of drugs (86,87). Furthermore, chicken embryos are a well-established and widely used model in virus research and vaccinology (88,89). A few studies have

demonstrated the ability of ZIKV to replicate potently in infected chicken embryos (90,91), and causing a similar pathology as seen in humans: virus replication in various organs, microcephaly and enlarged ventricles (91,92).

Overall, many complex models have been established and are constantly being developed to study ZIKV infection and facilitate drug and vaccine development, each with their own set of advantages and limitations.

1.5 COMPOUND PROFILING

Despite many new and highly sophisticated disease models, a prevailing challenge in drug discovery is to identify the molecular target(s) and pinpoint the specific effect of the drug of interest on cellular pathways, especially if it is a previously uncharacterized molecule. Many drugs also have poly-pharmacological effects, affecting not only one specific target, but manipulating diverse cellular pathways. Recently, new approaches like thermal proteome profiling (TPP), have opened new ways of compound characterization. TPP enables the monitoring of changes in protein thermal stability introduced through protein-drug interaction across the proteome using quantitative mass spectrometry to quantify these changes and thus facilitating the identification of drug targets (93).

1.6 THE DIAGNOSTIC LANDSCAPE FOR VIRUSES AND CHALLENGES IN THE DIAGNOSTICS OF EMERGING VIRUSES

To fight a newly emerging pathogenic virus, timely availability of accurate diagnostic tools is indispensable. From a single-patient perspective, knowledge about what causes the patient's disease is needed to guide treatment and facilitate recovery. From a societal and public health perspective, implementation of effective disease surveillance and control is impossible without widely available, sensitive and specific tests. Generally, there are two approaches to virus diagnostics: direct and indirect diagnostics.

1.6.1 Direct virus detection methods

Direct diagnostic tools detect virus material, like viral proteins or its nucleic acids in patient samples. These approaches can be based on various different technologies explained in the following paragraph.

1.6.1.1 Electron microscopy

The most immediate virus detection method is detection of the virus directly from a patient sample using electron microscopy. However, especially in samples with a low virus load, it might be necessary to enrich the virus by isolation in cell culture to increase the sensitivity of the method (94).

1.6.1.2 Virus isolation

For a long time, virus isolation from patient samples was the gold standard method in virological diagnostics. In this approach, the patient sample is cultured on cells, tissue or even

in animals until a cytopathic effect (CPE), virus induced cellular destruction, is detected. After detection of CPE follow up tests are needed to determine which virus has been isolated. If there is a clear suspicion based on the clinical presentation of the patient, the follow-up can be targeted using qPCR or antigen tests. If the pathogen is unknown, a non-targeted method like electron microscopy or next generation sequencing (NGS) is needed to determine which virus has been isolated. The virus strains isolated from patient samples are also a valuable tool in research to study pathogenicity and compare virulence between various virus strains (94).

However, there are several drawbacks to virus isolation. It is highly time consuming, taking several days at the least. Furthermore, virus isolation is subject to significant biological variation, and depends on the permissiveness of cells or tissue to the virus in question. To add another layer of complication, not all viruses cause CPE, making a negative result hard to assess without resource-heavy follow up-tests like NGS. Furthermore, especially in the case of emerging RNA viruses, virus isolation usually entails access to expensive laboratory infrastructure, skilled personnel, and high safety measures to contain the biorisks associated with experiments of this kind. Oftentimes these resources are not available in low- and middle-income countries.

All these drawbacks question the suitability of virus isolation for primary diagnostics, where the aim is a timely and precise answer. Likewise, in the past decade, the emergence of ever new and more sensitive molecular virus detection methods started to replace virus isolation in routine virus diagnostics. Nonetheless, especially for newly emerging infections with unknown pathogens, isolation oftentimes remains the crucial first step to discover a new pathogen and be able to characterize it.

1.6.1.3 Virus antigen detection

Immunoassays in either liquid or solid form are a frequently used technology to detect virus antigens in patient samples or as follow up test after virus isolation. The principle is capturing virus antigens from the patient sample with an antibody, which can be either in solution or attached to a membrane. In a second step, the complexes formed are identified by a detection reaction.

In the last two decades, technologies to detect nucleic acids have become more accessible and affordable, and especially qPCR has become the gold standard in detecting virus diagnostics (94). qPCR is a highly specific diagnostic tool, has a fast turn-around time of only a few hours from sample arrival to result and offers opportunities to react quickly to virus changes, like the emergence of new strains (94). In recent years the portfolio of nucleic acid detection methods in virus diagnostics has been complemented by isothermal amplification techniques like loop-mediated isothermal amplification (LAMP) (95) or rolling-circle amplification (RCA) using padlock probes (PLPs) (96). PLPs are linear, single stranded DNA oligonucleotides which consist of complementary target arms (15-20 nucleotides in length) and a longer, non-hybridizing backbone (40-50 nucleotides in length). Binding of the target arms to their respective target results in a circle which is linked through DNA ligation. After circulization,

the probes are amplified using a DNA polymerase. Digesting the amplification product and repeating the ligation and amplification step are called Circle-to-Circle amplification (C2CA) and increases sensitivity. Detection of the rolling circle products is performed using fluorescent labelling. Advantages of the method include isothermal amplification and high specificity, because perfect alignment of hybridization arms and ligation site are required (97,98).

Another trend seen in the past ten years was an increasing number of multiplex tests which facilitate the detection of pathogens usually grouped by the clinical presentation, like pathogens causing respiratory infections or meningitis. Most of these devices are based on detection of nucleic acid detection (99). While this approach is mostly limited to identifying previously known pathogens, it helps to streamline and speed up diagnostics. Several of these multiplex tests are increasingly easy to handle and could even potentially be used by trained staff as point-of-care diagnostics (100).

Furthermore, the increasing affordability of sequencing technologies opens up even more possibilities to use these technologies. In the ongoing COVID-19 outbreak setting, these tools have proven to be invaluable to determine how the virus evolves into new lineages and coupling this information to new phenotypic features like increased transmissibility (101).

However, an essential disadvantage of any method using nucleic acid detection, is that it cannot predict infectivity. In this context, the high sensitivity of the method is a drawback. It has been shown that viral RNA can be detected in various patient samples long time after infection, and the significance for patient outcome and infectivity are often unclear (102,103). Methods like virus isolation can provide insights on infectivity.

1.6.2 Indirect detection methods

Indirect diagnostics are based on the immune reaction triggered by virus infection. Typical assays used to determine the existence of antibodies towards a virus in patient serum are enzyme-linked immunosorbent assay (ELISA) or immunoblots (94). As a common principle synthetically produced virus antigens are used to capture potential antibodies present in the patient sample and the complexes formed between the virus proteins and patient antibodies are detected by a detection reaction (94). With the increasing recognition of the role of cellular immunity induced by T helper and cytotoxic T cells towards viruses, assays are being developed to measure cellular immune responses. In enzyme-linked immunosorbent spot (ELIspot) assays, patient cells are stimulated by virus antigens. The cytokines released by the cells as a result of the stimulation are captured on a membrane coated with antibodies for cytokines, and the complexes are then visualized by a detection reaction. Currently ELIspot assays are not part of routine diagnostics for viral diseases but are used to assess vaccine efficacy and for research purposes (104–106).

ELISAs, immunoblots and ELIspot assays are highly dependent on the peptides and proteins used as antigens to stimulate cells or capture antibodies. Especially in the beginning of a pandemic, when there is little knowledge about a virus, the production of highly specific

antigens might be difficult to achieve, leading to sensitivity issues in the detection. In this context also the variability in the human immune response pose additional challenges (107).

Immunofluorescence (IF) assays on the other hand are very resource heavy, since an IF microscope is needed for the assay. If there are no commercial IF assays available, similar resources are necessary as described above for virus culture, once again disadvantaging low-income settings.

Furthermore, cross-reactivity of antibodies between different viruses of the same family is a known issue in all indirect assays and has been shown for example for members of the Flavi- and Coronavirus families (108–110). Cross-reactivity affects test specificity and can make a specific diagnosis difficult, especially in geographical regions where many different viruses from the same family are circulating.

All in all, the virus diagnostics field has seen a technological revolution in the past three decades and moved from cell-based virus isolation as gold standard to broad application of highly specific molecular diagnostic methods. However, continuous research and development is needed to improve the methods available today and provide new innovative ways for virus detection to be prepared for future outbreaks.

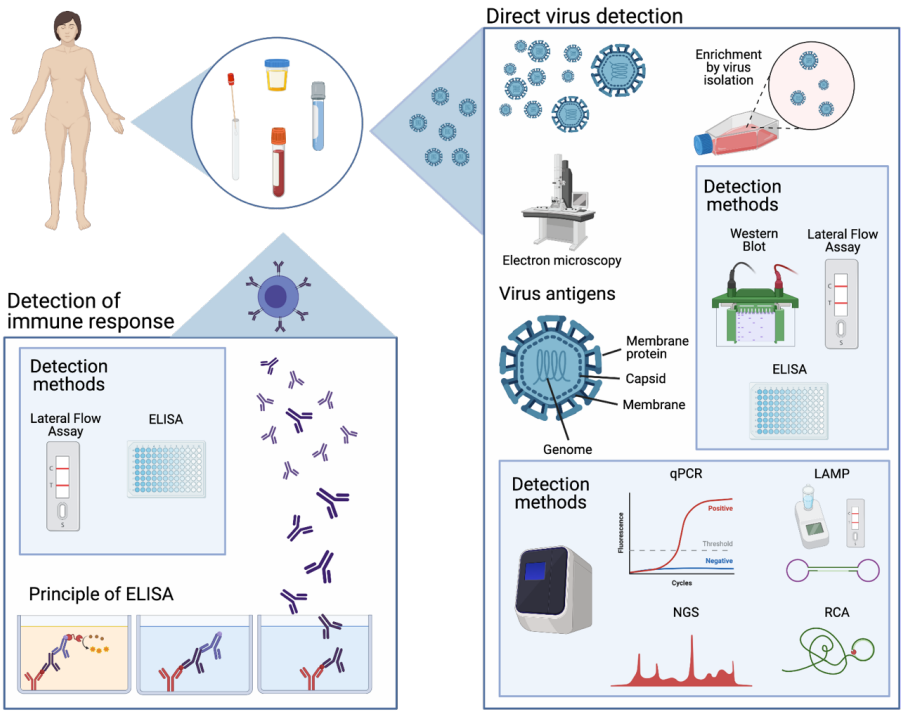

Figure 4: Methods used for virus detection

2 DOCTORAL THESIS

2.1 RESEARCH AIMS

The aim of this thesis was to show how various cellular and organoid models can be used to identify and characterize new antivirals and develop new diagnostic tools.

Specific contribution of each paper to the aims

- **Paper I - Novel broad-spectrum antiviral inhibitors targeting host factors essential for replication of pathogenic RNA viruses**
  - Use of an image-based phenotypic antiviral screening assay to identify host-targeting antivirals
  - Application of the image-based phenotypic antiviral assay to determine the antiviral activity of the newly identified antivirals against Coronavirus 229E (CoV 229E) and SARS-CoV-2

- **Paper II - Broadly active antiviral compounds disturb Zika virus progeny release rescuing virus-induced toxicity in brain organoids**
  - Transfer of the image-based phenotypic antiviral screening assay to a different cell-virus-system
  - Application of the image-based phenotypic antiviral assay in ZIKV infected glioblastoma cell line U87 to determine the therapeutic window of newly identified antivirals
  - Use of an iPS cells derived 3D organoid model to study the antiviral effect of identified antiviral compounds

- **Paper III - Circle-to-circle amplification coupled with microfluidic affinity chromatography enrichment for in vitro molecular diagnostics of Zika fever and analysis of anti-flaviviral drug efficacy**
  - Use of ZIKV infected glioblastoma cell line U87 to establish circle-to-circle amplification coupled with microfluidic affinity chromatography enrichment (C2CA-μACE) as a new diagnostic tool
  - Use of PBMCs to benchmark C2CA-μACE relative to RT-qPCR and study antiviral drug efficacy

2.2 RESULTS

Recent outbreaks of pathogenic RNA viruses like SARS-CoV-2, EBOV or ZIKV highlight the need for new antiviral strategies as well as diagnostic tools to be better prepared for emergence of ever new pathogens. The papers included in this thesis feature the use of various cellular and organoid models to facilitate the development of host-targeting antivirals and new diagnostic tools against emerging viruses.

2.2.1 Paper I - Novel Broad-Spectrum Antiviral Inhibitors Targeting Host Factors Essential for Replication of Pathogenic RNA Viruses

In this study, an image-based phenotypic antiviral screening assay was established using Hazara virus (HAZV) infected SW13 cells. HAZV is an RNA virus, which is not pathogenic for humans and can be used at biosafety level two laboratories but serves as a model for Crimean-Congo hemorrhagic fever virus. The main readouts of this assay were cell survival measured by nuclei count, number of infected cells as share of DMSO treated controls and virus progeny release, determined by titration of collected supernatants on fresh cells. Using the screening assay, 425 compounds from an in-house library of host targeting small molecular inhibitors were tested for their antiviral activity, revealing two close analogues TH3289 and TH6744 as hit compounds. The antiviral activity of both compounds against HAZV was confirmed by determining the antiviral effect in a dose-dependent way and showing a therapeutic window between the antiviral effect on the viral titer and cell viability. Moreover, both new antiviral compounds were confirmed to have a broad-range antiviral activity against several pathogenic RNA viruses including SARS-CoV-2, EBOV and Crimean-Congo hemorrhagic fever virus.

Originally both TH3289 and TH6744 were designed to inhibit human 8-oxoguanine glycosylase 1 (OGG1). However, the antiviral activity of the compounds was shown not to depend on their ability to inhibit OGG1. As a next step, to investigate the target of the new antiviral compounds in more detail, thermal protein profiling was performed with TH6744. Using this approach, the compound was shown to affect proteostasis pathways and disturb interactions between cellular HSP70 complex and viral proteins.

2.2.2 Paper II - Broadly Active Antiviral Compounds Disturb Zika Virus Progeny Release Rescuing Virus-Induced Toxicity in Brain Organoids

In the second study, the previously established image-based phenotypic antiviral screening assay was transferred to test the antiviral activity of in-house antiviral compounds in Zika virus infected cells. A comparison between the antiviral screening from **Paper I** revealed the same compounds to be active against both HAZV and ZIKV, confirming the broad antiviral activity of the compounds. Next, the therapeutic window of the top-hit compounds was demonstrated in several cellular models of ZIKV infection by comparing the dose-response analysis of the antiviral effect and cellular toxicity.

To study the antiviral activity of the compounds in a more physiological model of ZIKV infection, a 3D organoid model was established using human iPS cells. The novel antiviral compounds showed an antiviral effect on both infected organoids and viral progeny production and additionally reversed ZIKV induced neurotoxicity.

Subsequently, the compound's mechanism-of-action was studied by investigating intra- and extracellular ZIKV RNA levels over time and doing various time-of-addition experiments. Interestingly, ZIKV RNA levels were not impacted by treatment with TH6744. Instead, a rapid reduction of progeny release was detected even upon short treatment of 2h at late stages of infection. To investigate this finding further, the budding efficiency was studied but revealed no difference in the reduction of infectious virus particles between intra- and extracellular ZIKV particles. Altogether, the mechanism of action studies narrowed the mechanism-of-action down to impairment of formation of new virus particles in the late ZIKV lifecycle steps. It also revealed that TH6744 does not only rescue ZIKV induced pathologic phenotypes in cells and organoids, but also reduces virus transmission.

In summary, **Paper I** and **Paper II** highlight the value of various cellular and organoid models in the development and characterization of new host-targeting broad-spectrum antivirals.

2.2.3 Paper III- Circle-to-circle amplification coupled with microfluidic affinity chromatography enrichment for in vitro molecular diagnostics of Zika fever and analysis of anti-flaviviral drug efficacy

In **Paper III,** the development of a new diagnostic tool for ZIKV nucleic acids is described. Sequences from ZIKV genetic material were detected from ZIKV cDNA using padlock probes, targeting various region of the ZIKV genome, in particular regions coding for capsid (C), precursor membrane (PrM), envelope (E) and non-structural proteins (NS). Detection was followed by two rounds of Circle-to-Circle Amplification (C2CA) combined with a microfluidic affinity chromatography enrichment (μACE) platform.

In a first step the limit of ZIKV RNA detection (LoD) was determined for both a single round of rolling circle amplification (RCA) and two rounds (C2CA) of RCA using a dilution series of a single ZIKV synthetic ssDNA. A comparison of RCA and C2CA performance revealed C2CA to be superior and decrease the LoD by 3-fold, to be between 10^3 to 10^4 copies/mL. Consequently, C2CA was chosen for further method development and validation.

Next, ZIKV RNA extracted from cell culture supernatant from *in vitro* infected U87 was used to find an optimal method to determine the fluorescence of the C2CA products. Altogether 12 padlock probes were used to amplify the ZIKV RNA from the supernatants of infected U87 cells, and thereafter fluorescence was measured using either glass slides or μACE. Both measurements led to an effective detection of ZIKV compared to the negative controls. However, μACE provided a better signal to noise ratio through a higher selectivity of the μACE towards RCPs compared to artefacts like non-specific fluorescent clusters or debris. Thus, μACE chosen as the detection method of choice for the following experiments.

Subsequently, to benchmark C2CA-μACE against a gold standard diagnostic method, viral RNA obtained from *in vitro* infected PBMCs was quantified by both RT-qPCR and C2CA-μACE, revealing a good correlation between both methods as depicted by a Pearson correlation coefficient (*r*) above 0.8. Subsequently, the method was validated in *in vitro* infected PBMCs from three healthy donors infected with ZIKV and treated with Ribavirin, an FDA approved antiviral drug. The subsequent reduction in ZIKV RNA by 1-log detected by RT-qPCR was mirrored by the C2CA-μACE method.

In summary, this study shows a promising approach to develop a highly sensitive, simple and cost-effective point-of-care viral diagnostics tool. Moreover, it is demonstrated how using cell lines and PBMCs can facilitate development of diagnostic tools for emerging viruses.

2.3 DISCUSSION

The WHO has long been advocating for increased research and development efforts, to create new antivirals and diagnostic tools for diseases with epidemic potential (111).

A major obstacle to the development of new antivirals is the lack of good models to study viral diseases. Developing new approaches, refining and improving models used in virus research is critical to advance drug discovery. This thesis studied the use of various cell models in antiviral drug discovery and diagnostics development, highlighting advantages and disadvantages of the various models.

2.3.1 Using phenotypic screening as an approach to antiviral drug-discovery

In **Paper I** we present an image-based phenotypic screening assay using the adrenal gland small cell carcinoma cell line SW13 and HAZV. In contrast to many other screening assays published, infectious virus is used in the assay presented, and thus our system allows to study the effect of the tested compounds on all virus lifecycle steps. There is a plethora of approaches to antiviral screening, ranging from screening systems using virally induced cytopathic effects as a readout (112,113) to screening using replicon systems (114,115). Very often these replicon systems are unable to produce infectious viable virus progeny (116), making them especially interesting in the context of emerging RNA viruses, many of which entail handling at facilities with high biosafety. A drawback of using replicon systems for antiviral screening is that without production of infectious virus progeny, not all steps of the virus life cycle can be examined, making the antiviral screenings blind towards drugs that impair virus progeny release.

The assay design utilized in **Paper I** allows to check for inhibition of all virus life cycle steps, while in addition mimicking the real-life situation of treatment following infection. While there are previous reports of image-based screening for antivirals using infectious virus in cell lines (117,118), in most published studies, the cells were pre-treated with the drugs before virus infection and only primary virus infection was studied not virus progeny release (117,118). This approach biases the assays towards drugs that inhibit the virus in early steps of the virus life cycle, like viral entry or translation and RNA synthesis.

In **Paper II** the adaptability of the image based phenotypic screening approach is highlighted by a transfer of the assay from HAZV infected SW13 cells to ZIKV infected U87 cells. A versatile assay is important in the development of drugs against emerging viruses, to be able to adapt quickly, and transfer the assay to a new virus-cell-system for drug development or testing against previously unknown viruses.

Despite the advantages mentioned above, the image-based phenotypic screening approach used in **Paper I** and **Paper II** has some limitations. One inherent limitation comes with the usage of antibodies to detect the different viruses. Firstly, this detection method is highly dependent on the availability of specific antibodies with low background. Especially for newly emerging viruses the development of good tools to use in an image-based assay can take a while and limit

immediate application of the assay. Furthermore, different viral proteins are expressed at different timepoints of infection in different quantities (119). Thus, during assay development, the assay needs to be well controlled, for example by using antibodies against several viral proteins and comparing them, as well as performing an infection-time and -dose kinetic study to determine optimal experimental conditions. To exclude antibody-based bias, immunofluorescence staining of ZIKV NS1 and Capsid proteins was compared in **Paper II** and both antibodies were demonstrated to have the same sensitivity for detecting ZIKV-infected cells.

By choosing imaging as a readout, the phenotypic screening assay presented in **Paper I and II** provides a single-cell resolution, enabling studying the effects of virus infection and treatment on each cell. But, in the studies presented, only nuclei count determined by DAPI staining is used as a readout for cellular toxicity. While DAPI staining is comparably easy to determine and analyze, a more thorough assessment of the host cells could provide insights into the mechanism of action of compounds on the host cells. Morphological profiling of cells by new techniques like Cell Painting, can maximize the available information extracted from image based screenings and foster antiviral drug discovery (120,121).

2.3.2 Organoids as models to study Zika virus infection

In **Paper II** an iPS cell derived 3D brain organoid model is established and used to validate the top hits compound identified in the image-based phenotypic screening assay. 3D models have been shown to be advantageous over 2D cell culture models in mimicking some of the brain's architecture and complexity (75). In the context of neurotropic infections, 3D brain organoids are valuable tools to investigate virus spread and infection dynamics over time, viral tropism, virulence, and even long-term effects of virus infection.

In 3D models, infection is much less efficient than in 2D models, since only certain surface parts of the organoid are accessible for infection. To take this into consideration, in **Paper II** infection time was extended to 24h to establish ZIKV infection in the organoid models. Moreover, there was an increase in progeny production with increasing dose and time of infection, reflecting infection spread through the organoids as more cells got infected over time.

3D brain organoids significantly contributed to connecting ZIKV neurotropism to fetal microcephaly after maternal ZIKV infection during pregnancy (122). In the study presented here, ZIKV neurotoxicity was shown through organoid viability reduction as well as reduced production of ZIKV progeny by organoids at the highest infection dose at 7 dpi. The neurotoxicity enabled highlighting the phenotypic rescue upon treatment with our compounds, illustrated by an increased viability, reduction of primary infection and ZIKV progeny production. However, further characterization of ZIKV neurotoxicity and its rescue seen in our model would be highly interesting. Cugola et al. and Qian et al. were among the first to describe that ZIKV causes apoptosis in neuronal progenitor cells, limiting their viability and impairing organoid growth (123,124). It would be of interest to see the effects of our treatments on

apoptosis and cell proliferation, and especially if certain cell-types are particularly affected by ZIKV infection and treatment.

A problem faced in **Paper II** was the previously described variability between organoids (125): while in some organoids ZIKV infection led to a strong reduction in viability and size, other organoids infected in the same culture dish remained seemingly unaffected. This unique effect of ZIKV on each organoid entailed a high intra- and inter-experimental variability. Furthermore, it made it especially difficult to study changes in organoid size, without following up on each organoid separately. On the other hand, this variability also reflects the real-life setting, since only less between 2.5-5% of babies born to mothers with ZIKV infection during their pregnancy develop birth defects (26,27). In the rest of the cases, the fetuses do not suffer from microcephaly, despite a maternal ZIKV infection during pregnancy.

Even if 3D brain organoids come closer to mimicking the human brain than 2D cell cultures, some important features are still missing. Missing parts include key determinants of infection in an organism like the blood flow and especially an immune reaction. This drawback of the model became apparent in the studies of ZIKV infection in human brain organoids presented here, as it was not possible to detect cytokine secretion in ZIKV infected organoids at 1 and 7 dpi (data not shown). However, there are various promising approaches to improve brain organoid models to include these and other features and make the method even more appealing and comprehensive. Recently there were reports of organoids containing microglia and responding to ZIKV infection by increased expression of pro-inflammatory genes (126) and of organoids containing a vasculature like system (127). Furthermore, organoids containing cells from the choroid plexus and producing cerebrospinal fluid were developed, enabling future investigations of drug-neurotoxicity and the blood brain barrier (128).

Altogether, 3D brain organoids already are a valuable tool in antiviral drug development, but it remains interesting to see if the developments in the field will be fast and comprehensive enough for organoids to become a true alternative to for example animal studies.

2.3.3 Using cellular models to develop diagnostics

While in **Paper I** and **II**, cell-based models were used in antiviral drug discovery, in **Paper III** the focus was on facilitating diagnostics development.

In our study, using an established cell line for preliminary studies to optimize the C2CA-µACE system offered a reliable, readily available and easily reproducible source of samples. A similar approach has been previously used by other researchers in the establishment and pre-testing of new diagnostic methods (129).

After optimal conditions were determined, *in vitro* infected PBMCs were used to characterize the method more in detail. An advantage of this approach is that it mimics wide parts the real-life scenario of human infection. ZIKV replication in human PBMCs before collection of supernatants for RNA extraction and detection by qPCR and C2CA-µACE system is comparable with what happens in patients with exception of the mosquito bite. The similarity

of the systems is underscored by previously published reports of viremia levels in patients of around 10^4 copies/mL (130,131). Thus viremia levels in patients correspond well to the ZIKV levels detect in supernatants from infected PBMCs in **Paper III**. Alternative approaches used human material from healthy donors like plasma or urine spiked with ZIKV RNA (132). While the amount of RNA spiked into the sample can be very well controlled, it is also a very artificial approach compared to the approach chosen in **Paper III**.

To summarize, while cell culture and *in vitro* infected PBMCs will never replace true patient samples for the validation of diagnostic tests, they can be a way to make diagnostics development faster and more cost-effective. Furthermore, using *in vitro* infected blood cells can help research groups without access to patient samples to participate in diagnostics development. Still, before use in the clinics, the approach would need to be validated thoroughly using patient samples with different amounts of virus to determine sensitivity, specificity and limit of detection of the C2CA-µACE system.

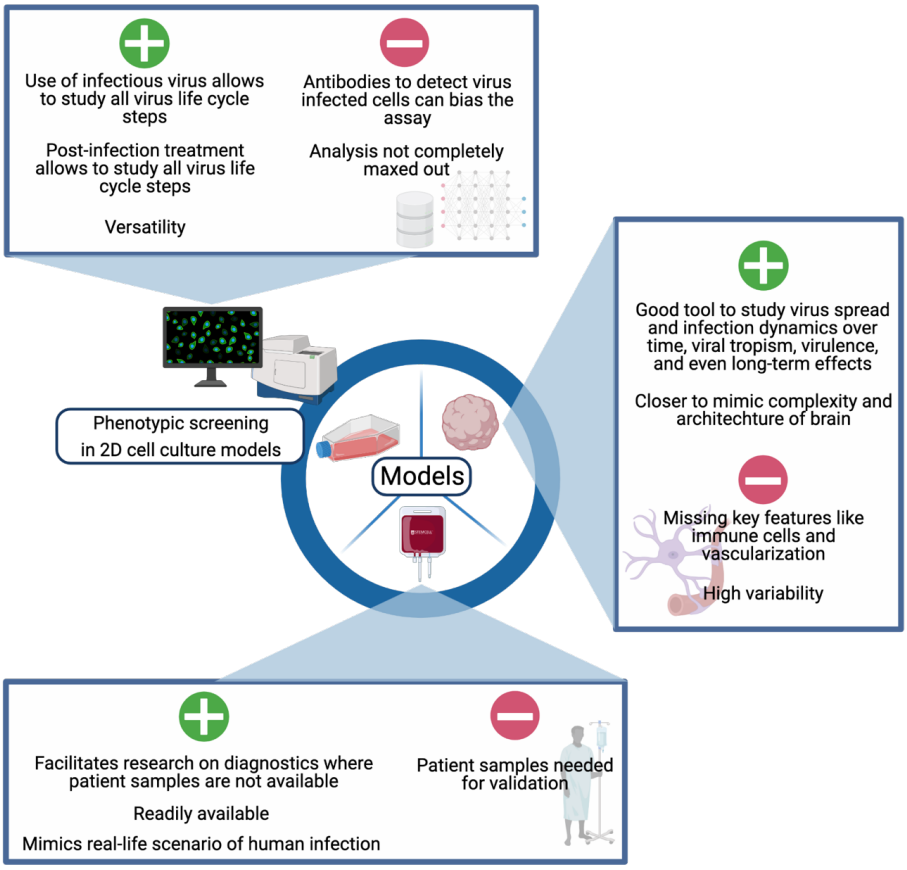

Figure 5: Graphical summary of discussion

2.4 CONCLUSIONS AND FUTURE PERSPECTIVE

Several pandemics occurred in the past decade including the biggest Ebola outbreak recorded so far in Western Africa from 2014-2016, the ZIKV epidemic in South America between 2015 and 2016, and the currently still ongoing global pandemic of COVID-19. Surprisingly, despite many warnings and predictions, each of these pandemics caught us off guard, revealing that increased efforts are needed in pandemic preparedness, including drug and diagnostics development, to be able to react to future threats faster and more efficiently.

In the phenotypic screening assay used in **Paper I** and **II** many terabytes of data were generated. To facilitate analysis, data management programs like KNIME were used. However, this was only a small step towards automated data analysis and many more tools like artificial intelligence (AI), machine learning or deep learning are available and are constantly being developed. As science generates more data than ever before, AI and deep learning will play an increasing role in drug discovery and development. Not only will it enhance data management and analysis of high content assays including imaging assays, but AI can provide completely new approaches and impulses to drug discovery. *In silico* screens were among the first in the current COVID-19 pandemic to screen billions of compounds and predict compounds with potential antiviral activity against SARS-CoV-2 (133,134). While *in silico* screens cannot substitute for biological assays, they can help to pre-screen a huge number of compounds and narrow down potential antiviral drug candidates, or to identify potential drug targets. With enough computational capacity, several billions of compounds can be screened *in silico*, a scope which can never be met in biological assays and opens previously unimagined opportunities for drug screenings. Blended approaches using machine learning and deep learning in several steps of the drug discovery process from target identification, study of structure-activity-relationships or analysis of assay results will enhance and speed up the drug discovery process and make it more cost-effective, hopefully resulting in new, broadly active antivirals in the future.

Paper II highlights the use of human brain organoids in drug development as a secondary model to follow up on top hits from a compound screening. However, the limitations of the model like the lack of an immune response prohibited the use of the model for further in-depth studies of the compounds and their effect on ZIKV infection in brain organoids. Thus, to increase the value of organoids in drug discovery, some key developments will be needed. This includes even more neuronal cellular complexity, as well as successful implementation of immune cells to be able to assess the immune response. Furthermore, introduction of endothelial cells and implementation of microfluidics in the models can simulate a blood flow in the organoids, promoting long-term survival of organoids and enabling to simulate the distribution of substances and pathogens within the organoids. While collaboration is important in research in general, for highly specialized models like organoids, collaboration is key to push new developments forward and improve techniques. Sharing protocols and resources as well as creating platforms and biobanks can promote the developments described in this paragraph, lead to a wide availability of organoids and increase their use in drug discovery.

To enable diagnostics early in an outbreak, new and innovative approaches towards virus diagnostics are needed. **Paper III** highlights the development of a sensitive and versatile assay to detect ZIKV RNA using RCA. Additionally, the assay has the potential to be scaled down towards point-of-care-testing. Taking into account that many viruses with pandemic potential (re)-emerge in resource-poor regions with limited access to high-end testing facilities and machinery, the development and use of affordable and easy-to-use point-of-care-testing is essential. More point-of-care-testing will not only expand access to diagnostics, but also offer more opportunities to detect and contain outbreaks on a regional level through increased surveillance of the pathogen landscape.

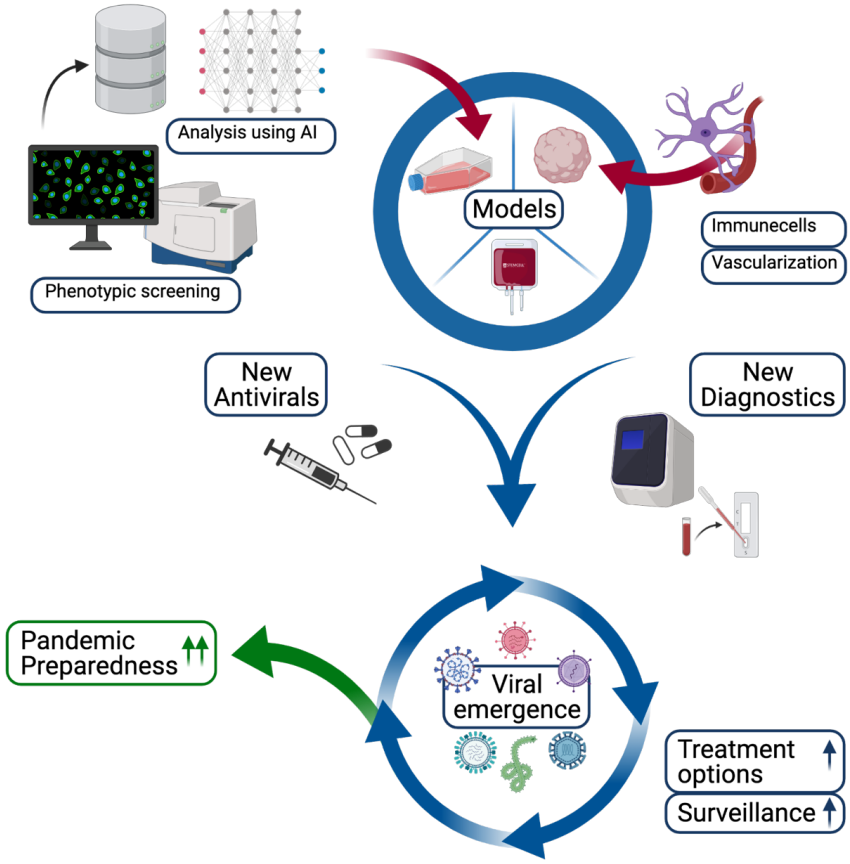

Figure 6: Implications of development for pandemic preparedness

A key experience during my Ph.D. was, that research is becoming increasingly multidisciplinary and collaborative. All projects presented in this thesis were carried out in teams of scientists with different backgrounds both within the Helleday Lab but also as collaborations with other labs. Increasingly, experts from many different fields are working together and sharing expertise. Especially fields like drug discovery or diagnostics

development, which are on the cutting edge of many different sciences, like chemistry, biology, pharmacology, biotechnology, veterinary and human medicine, will benefit but also depend on increasing interdisciplinarity and collaboration and strengthening networks. A success story highlighting the importance of collaboration as well as previously built infrastructure and capacity in the diagnostics field, is the development of a diagnostic protocol to detect SARS-CoV-2. The first known case of SARS-CoV-2 infection was traced back to 17th November 2019, a first viral genome sequence was publicly available on 7th January 2020, and a first diagnostic test was provided to the WHO on 13 January 2020 by a group of researchers (135). This success was enabled by collaborations between European and Chinese researchers, established in response to previous outbreaks, as well as the European Virus Archive. Through joint efforts, enough clinical samples and reference RNA sequences could be tested to evaluate the qPCR test for specificity and sensitivity and deploy it in record time. To tackle the problems of the future, more collaboration will be needed than ever, and strengthening communication and networks of scientists from different fields should be a priority.

Altogether, emergence of unknown viruses and re-emergence of known viruses like Ebola will be a challenge for decades to come. With each outbreak there is a gain of knowledge and skills, improving the ability to cope with the next outbreak. Still, pandemic preparedness needs to be actively developed, including innovative platforms to contribute to fast vaccine development and distribution, new broad-spectrum antivirals and diagnostic tools. This work shows how cell culture tools of varying complexity can be used in the search for novel drugs and diagnostic methods. While none of the models is perfect, each of them comes with their own merits and challenges, with unique application possibilities. It will be key to understand better how to combine and use them in the most beneficial way in combination with AI and other new technologies.

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